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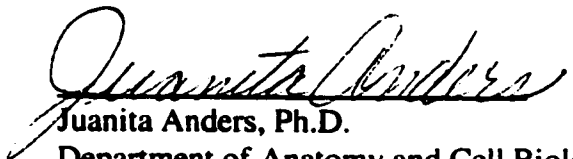


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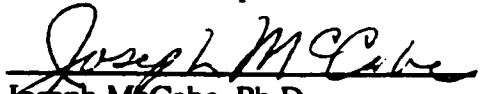
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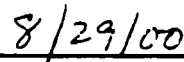
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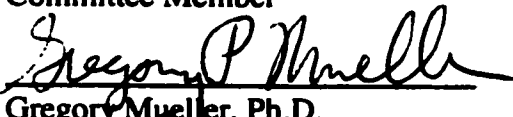
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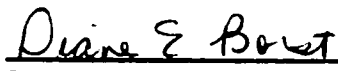

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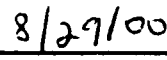

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A handwritten signature in black ink, reading "David Alan Zemo". The signature is fluid and cursive, with the first letters of each name being capitalized and prominent.

David Alan Zemo
Department of Anatomy and Cell Biology
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Abstract

Title of Dissertation: Osmotic Stress Induces Transcriptional Changes in Vasopressin and Vasopressin 1b Receptor Gene Expression

David A. Zemo, Doctor of Philosophy, 2000

Dissertation directed by: Joseph T. McCabe, Ph.D.
Professor
Department of Anatomy and Cell Biology

Arginine vasopressin (AVP) plays a critical role in the regulation of mammalian salt and water homeostasis. To further define central nervous system adaptation to osmotic challenges, transcription of AVP and vasopressin 1b receptor ($V_{1b}R$) genes by magnocellular neurons of the hypothalamus and epithelial cells of the choroid plexus was studied using *in situ* hybridization. Compared to animals given a single injection of normal saline, animals that received a single injection of hypertonic saline had increases in AVP heteronuclear RNA (hnRNA) after 15 and 30 minutes. Animals given an injection of hypertonic saline followed by a second injection of hypertonic saline (H-H) had an increase in AVP hnRNA levels that were equivalent to the response seen after a single hypertonic saline injection. Levels of AVP hnRNA after H-H were greater than the levels detected in animals given an injection of normal saline followed by a second injection of hypertonic saline (N-H). This is the first study to show repeated exposure to hypertonic saline causes an immediate, robust, and reproducible increase in vasopressin gene transcription. These results suggest there is a correlation between increased neuronal firing rate and vasopressin gene transcription. We also studied long-term adaptation to an

osmotic challenge. Compared to rats maintained on tap water, salt-drinking rats had increased levels of AVP and $V_{1b}R$ mRNAs in the supraoptic and paraventricular nuclei, and in the choroid plexus. The increase in AVP and $V_{1b}R$ mRNAs in the SON and PVN as a result of plasma hyperosmolality may indicate the intranuclear release of AVP has a role in the autoregulation of magnocellular neuron activity. The role of AVP in cerebrospinal fluid formation remains to be elucidated. However, the increase of AVP and $V_{1b}R$ mRNA in the choroid plexus suggests the involvement of AVP in the regulation of brain water content and cerebral edema. Taken together, these results demonstrate coordinated transcriptional control in hypothalamic neurons and epithelial cells of the choroid plexus. Changes in gene regulation for a receptor and its binding peptide occurred in two structures that have crucial, complementary roles in water homeostasis for the periphery and the central nervous system.

**Osmotic Stress Induces Transcriptional Changes in
Vasopressin and Vasopressin 1b Receptor Gene Expression**

by

David Alan Zemo

**Dissertation submitted to the Faculty of the Department of Anatomy
and Cell Biology Graduate Program of the Uniformed Services
University of the Health Sciences in partial fulfillment of the
requirements for the degree of Doctor of Philosophy 2000**

Dedication

To my loving wife, Sherryl Lee Zemo, thank you for reassuring me every time I encountered a fork in the road. The completion of this work would be meaningless to me if you were not by my side to share it. Your sacrifice and support the last seven years transcend the boundaries that measure all spouses.

To my son, Porter Alden Zemo, although your birth interrupted the writing of this dissertation, it was also another piece of the puzzle completing my life. Someday I hope my dedication of this work to you will inspire your motivation and compassion as your birth has for me.

To all members of the research community, from graduate students to professors and from laboratory workers to presidents of universities, may the devotion of so few trying to make the universe better for every living thing never go unnoticed.

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Dr. Gregory Mueller, your expertise in academic training and writing is a model I will look up to the rest of my career. Even though your time was limited, you gave all I needed to

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Chapter One

The Anatomy and Physiology of Vasopressin

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Vasopressin Gene Structure and Hormone Production

Vasopressin is a neuroendocrine hormone secreted primarily by neurons of the hypothalamo-neurohypophyseal system. The cell bodies of this system are concentrated in two nuclei of the hypothalamus, the supraoptic nucleus (SON) and the paraventricular nucleus (PVN), and their axons course through the hypothalamus and terminate in the posterior pituitary gland. Vasopressin messenger RNA (mRNA) is translated to form a propeptide consisting of the nonapeptide hormone arginine vasopressin (AVP), carrier protein neurophysin II attached to the carboxyl terminal end of AVP, and a glycoprotein on the carboxyl terminal end of the propeptide (Brownstein et al., 1980; Schmale et al., 1983). The propeptide is packaged within the Golgi apparatus into neurosecretory granules. Cleavage of the propeptide occurs within the neurosecretory granules while in the cell body, during axonal transport, and within the nerve terminal resulting in the hormone and neurophysin (Brownstein et al., 1980). In the posterior pituitary gland aggregations of granules, also known as Herring bodies, are stored. The stimulation of magnocellular neurons in the PVN and SON nuclei through afferent inputs evokes the release of AVP via exocytosis from nerve terminals in the posterior pituitary. The hormone diffuses across the extracellular space and into the plasma through fenestrated capillaries surrounding the nerve terminals. The release of AVP is coupled to vasopressin gene expression insuring that hormone stores are maintained in times of high demand (Sherman, 1996).

Vasopressin Physiology

Vasopressin is best known for regulating osmotic homeostasis through its water reabsorption effects on the distal tubule of the kidney (Richter, 1988). AVP has also been shown to mediate peripheral vasoconstriction (Weber et al., 1997), and it affects many adaptive behaviors, including sexual behavior, body temperature, memory, and stress (Bock et al., 1994; Ma et al., 1997; Born et al., 1998; Young, 1999). Recent evidence suggests neurohypophyseal hormones are important constituents of neural systems mediating parental behavior, intraspecies affiliation, and possibly infantile autism (Nelson & Panksepp, 1998; Insel et al., 1999; Young et al., 1999). In addition, AVP might be involved in the regulation of ion/water balance of the cerebrospinal fluid since the intraventricular administration of AVP causes an increase in brain water content (Raichle & Grubb, 1978; Doczi et al., 1982).

The magnocellular neurons of the hypothalamo-neurohypophyseal system are ideal for studying neuroendocrine gene regulation because of their robust and reproducible response to whole body fluid and electrolyte balance. In 1947, Verney showed that an increase in plasma osmolality caused an increase in the plasma concentration of vasopressin (Verney, 1947). Vasopressinergic neurons are classified into two types based on whether their initial firing pattern is irregular or phasic, and they respond differently to an increase in plasma osmolality (Brimble & Dyball, 1977). At first, irregularly firing neurons behave similarly to oxytocinergic neurons and increase their firing in a continuous manner, but after 10-15 minutes they become phasic. Phasic neurons increase their firing rates and their duration of activity. The increased firing of vasopressinergic neurons

results in the release of AVP and an increase in AVP gene transcription. In fact, a second injection of hypertonic saline sixty minutes after the first causes an even greater increase in neuronal firing rate and duration of activity (Brimble & Dyball, 1977).

In response to a stimulus, the ability to increase the activity of the translational apparatuses is limited. Thus, increases in specific mRNA pool sizes must be made in order to increase the biosynthetic capacity of a neuron. The magnocellular neurons of the PVN and SON increase their quantity of vasopressin mRNA due to many different osmotic stimuli including dehydration, salt-loading, and intra peritoneal (ip) injection of hypertonic saline (Burbach et al., 1984; Sherman et al., 1986; Murphy & Carter, 1990; Suemaru et al., 1990; Shoji et al., 1994; Amaya et al., 1999). A classical experimental method for strongly evoking neurohypophyseal system activity is salt-loading; providing rats with 2% saline. Salt-loading for six to ten days results in a two- to three-fold increase in AVP mRNA in the SON (Zingg et al., 1986; McCabe et al., 1990; Suemaru et al., 1990). Salt-loading for 14 days results in similar increases in AVP mRNA in the SON and PVN, and causes plasma levels of AVP to rise from 0.6 pg/ml to 5.2 pg/ml (Burbach et al., 1984; Van Tol et al., 1987).

While measurement of changes in AVP mRNA from salt-loading is important, evaluation of rapid alterations in gene transcription is more pertinent to further understanding neurohypophyseal hormone gene regulation. An ip injection of hypertonic saline, for example, rapidly changes plasma osmolality. Hypertonic saline administration increases plasma $[Na^+]$ from 140 mEq/l to 160 mEq/l within 15 minutes, and levels remain elevated (150 mEq/l) for as long as 180 minutes (Dunn et al., 1973; Brimble & Dyball,

1977; Shoji et al., 1994). Elevation of plasma osmolality triggers an increase in neuronal activity and the release of AVP from the nerve terminals in the posterior pituitary gland (Robinson & Fitzsimmons, 1993). Similar to changes in plasma sodium levels, plasma concentration of the hormone exhibits a rapid rise following osmotic stimulation, with levels decreasing in a manner that parallels plasma osmolality (Shoji et al., 1994). In contrast, AVP mRNA levels do not increase until 180 minutes after hypertonic saline administration, and remain elevated for at least 360 minutes (Shoji et al., 1994). A methodology that allows one to measure rapid changes in AVP gene transcription in the minutes after the initiation of plasma hyperosmolality is needed. As will be reviewed later, the measurement of heteronuclear RNA (hnRNA) levels may be a better tool for assessing changes in gene transcription.

Vasopressin Receptors

Characterization studies have employed combinations of agonists and antagonists to originally identify 3 types of vasopressin receptors: $V_{1a}R$, $V_{1b}R$, and V_2R . Vasopressin receptors are members of the G protein-coupled receptor family characterized by seven putative transmembrane domains (Burbach et al., 1995). Similar to other G protein-coupled receptors, the binding of AVP to the external ligand recognition site causes conformational changes of the receptor. These changes make putative internal phosphorylation sites more accessible to proteins within the cell that are involved in signal transduction pathways (Burbach et al., 1995; Ancellin et al., 1999). The activation, or repression, of intracellular pathways eventually influences the expression patterns of genes within the nucleus, and thus alters the function of the cell. The $V_{1a}R$, physiologically

involved in glycogenolysis in hepatocytes (Eugenin et al., 1998), and $V_{1b}R$, physiologically involved in modulating adrenocorticotrophic hormone (ACTH) secretion from corticotrophs in the anterior pituitary (Aguilera & Rabadan-Diehl, 2000), act via phosphatidylinositol hydrolysis and mobilization of intracellular Ca^{2+} (Antoni, 1993). The V_2R acts via adenylate cyclase producing cAMP, and is physiologically involved in modulating water channel function in the kidney (Knepper, 1997).

Investigators have cloned all three receptors and *in situ* hybridization has been used to localize their distribution in the central nervous system. $V_{1a}R$ mRNA was localized throughout the neuraxis (cortical areas, hippocampus, hypothalamus, and brainstem nuclei), the circumventricular organs of the brain (subfornical organ and area postrema), cerebral blood vessels, and the choroid plexus (Ostrowski et al., 1992, 1994). Although less widespread, $V_{1b}R$ mRNA was localized in corticotrophs of the anterior pituitary, dentate gyrus, hippocampus, and the choroid plexus (Burbach et al., 1995; Lolait et al., 1995). Originally, V_2R mRNA was not detected in the CNS (Ostrowski et al., 1992), but others recently localized V_2R mRNA in the cerebellum (Kato et al., 1995). Interestingly, V_2R mRNA was detected in epithelial cells and vascular endothelial cells of the choroid plexus in newborn rats, but was not detectable in the adult (Kato et al., 1995).

In 1995, a fourth AVP receptor was discovered, and the gene encoding this receptor is transcribed in the central nervous system (Ruiz-Opazo et al., 1995). Ruiz-Opazo found a novel *dual angiotensin II/AVP receptor*, and called it the *nasopressin* receptor (Ruiz-Opazo et al., 1995, 1998; Gonzalez et al., 1997; Ruiz-Opazo, 1998). There is no significant amino acid or nucleic acid homology between the V_2R and

nasopressin, with the exception of homology in the regions of both receptors where AVP peptide binds. To date, little further work has been performed to develop a better understanding of this receptor, although this month Hurbin and colleagues report that the mRNA encoding nasopressin is transcribed by neurons throughout the central nervous system (Hurbin et al., 2000). *In situ* hybridization demonstrated nasopressin was also present in the epithelial cells of the choroid plexus, the ependymal cells, and the pia mater. Nasopressin mRNA is also present in magnocellular neurons of the supraoptic and paraventricular nuclei.

In the same year that the nasopressin receptor was reported, a fifth AVP receptor, *vasopressin-activated, calcium-mobilizing protein* (VACM-1), was discovered by Burnatowska-Hledin and colleagues (Burnatowska-Hledin et al., 1995). This protein was shown to be a product of a rabbit renal expression library by injecting cDNAs into a *Xenopus laevis* oocyte system. A clone was found that released intracellular Ca^{2+} in response to AVP. It was then transfected into COS-1 cells and found to bind ^{125}I -labeled AVP (Burnatowska-Hledin et al., 1995). On Northern blots, it was found to produce two bands of 3.2 and 6.5kD in both the rabbit kidney and brain. It is thought to be different, but similar to both the V_1R and V_2R receptors in terms of what it binds. In 1998, North and colleagues found small cell carcinoma of the lung (SCCL) express V_{1a}R , V_{1b}R , V_2R , and VACM-1 (North et al., 1998). In 1999, Burnatowska-Hledin and coworkers reported finding human cell lines that transcribe VACM (Burnatowska-Hledin et al., 1999). Further analysis shows it is expressed in vascular endothelial cells of the brain, heart, muscle, ovary, and kidney medulla, but not in the gastrointestinal tract, aorta, or kidney

cortex. It is mentioned by Burnatowska-Hledin (Burnatowska-Hledin et al., 1995) that the VACM-1 protein appears to be a single transmembrane receptor, unlike the other VRs, which have the classic 7-transmembrane structure of G-protein receptors. Also, Burnatowska-Hledin mentions (Burnatowska-Hledin et al., 1999) that there is high homology to (and it is apparently a member of) the *cullin* gene family (Pause et al., 1997). The cullin gene, CeCul-1, is known to regulate "cell cycle transitions" of G₁ to G₀, while a paper by Pause and coworkers (Pause et al., 1997) reports another cullin gene, Hs-cul-2, is involved in the von Hippel Lindau tumor. Hs-Cul-2 acts like a tumor suppressor. This is a tumor that originates in the kidney. Two groups have recently characterized VACM. Burnatowska-Hledin and co-workers utilized immunohistochemistry to locate VACM proteins (Burnatowska-Hledin et al., 1999) and Hurbin and colleagues analyzed cellular expression of VACM mRNA by *in situ* hybridization (Hurbin et al., 2000). Surprisingly, the anatomical findings of these groups were very disparate. Burnatowska-Hledin states VACM is only present in vasculature of the central nervous system, while Hurbin reports VACM mRNA is not present in endothelial cells and was found in neurons exclusively. Further study must be performed to determine the reasons for such discrepancy.

Central Nervous System Functions of Vasopressin Receptors

The discovery of five different vasopressin receptors in the central nervous system has further provoked interest in understanding the role of centrally-mediated AVP functions. As noted earlier, vasopressin is known to have a role in the regulation of numerous neurological functions and behaviors. Investigators have employed receptor autoradiography to localize AVP binding in the central nervous system, and have related

neuroanatomical localization with its potential function (Freund-Mercier et al., 1988; Loup et al., 1991; Reghunandanan et al., 1998). Electrophysiological recording of the central effects of AVP upon neuronal activity of SON and PVN neurons has shown these neurons possess vasopressinergic autoreceptors (Ludwig, 1998; Dayanithi et al., 2000).

In pituitary corticotrophs, vasopressin receptors are regulated in an autoregulatory manner that could be similar to their function in the SON and PVN. During periods of chronic stress, vasopressin secreted by parvocellular neurons of the PVN ensures ACTH release by maintaining pituitary corticotroph responsiveness (Aguilera, 1994). In response to acute stress, pituitary corticotroph levels of the V_{1b} receptor are upregulated (Aguilera & Rabadan-Diehl, 2000). However, in response to chronic osmotic stress V_{1b} receptor levels are downregulated (Aguilera et al., 1994). In a similar manner, the application of hypertonic saline within the SON by microdialysis causes intranuclear (dendritic) release of AVP, and the release is blocked by a V_1/V_2 receptor antagonist (Wotjak et al., 1994). In Chapter Three, the regulation of V_{1b} receptor mRNA levels in SON and PVN neurons due to chronic osmotic stress will be reported.

The effects of osmotic stimulation upon vasopressin receptor function has been studied in the kidney and the CNS utilizing [3 H]AVP with receptor autoradiography. In the kidney, VR density decreased by 38% due to 72 hours of water deprivation (Steiner & Phillips, 1988). In addition, an injection of hypertonic saline decreased the number of VRs in the kidney by 32% (Landgraf et al., 1991). However, the injection of hypertonic saline did not change VR number in the hippocampus, septum, or amygdala.

Vasopressin's Role in Health and Disease

A classic endocrinological disorder resulting from disruption of vasopressin's urine concentrating ability is *diabetes insipidus*. This condition can arise from a failure to synthesize or release vasopressin, *central diabetes insipidus*, or from an inability of the kidney to regulate urine formation, *nephrogenic diabetes insipidus*, and both of these conditions exemplify Mendelian inheritance patterns. Families can transmit a heritable form of central diabetes insipidus that results from a mutation of the vasopressin gene. The mutation is most commonly a nucleotide base substitution within the region of the gene encoding the neurophysin, which causes an amino acid replacement (Repaske et al., 1997; Willcutts et al., 1999). The neurophysin moiety fails to assemble an appropriate tertiary structure. Mutation of nucleotide sequence encoding the signal peptide has also been described, where an amino acid substitution disrupts the ability of the signal peptide to be cleaved from the hormone segment (Repaske et al., 1997). Interestingly, mutation of the vasopressin gene is autosomal dominant in humans and results in the degeneration of neurohypophysial neurons in the hypothalamus (Repaske et al., 1997; Willcutts et al., 1999). In the Brattleboro rat, which inherits a mutated vasopressin gene arising from a single nucleotide deletion in the region encoding for the neurophysin (Schmale & Richter, 1984), expression follows a semi-dominant trait. Heterozygotic animals exhibit AVP plasma levels that are approximately 50% the levels of rats with no mutation.

A lack of sensitivity to vasopressin's action upon the distal renal nephron can also be inherited. In this case, the individual may synthesize and secrete AVP, but the renal V_2R , or water channels regulated by V_2R signal pathways, exhibit a mutation.

Inherited nephrogenic diabetes insipidus arising from mutations of V_2R is an X-chromosome-linked disorder, and therefore is a more frequent condition in males. Mutations of the aquaporin-2 (AQP2) gene are inherited as either an autosomal recessive or dominant condition (Knoers & Monnens, 1999). AQP2 is a water channel expressed by principal cells in the apical segment of the collecting duct, as well as by collecting duct cells of the renal inner medullary region. Activation of signal transduction pathways increases water permeability of the apical membrane by activation of aquaporin, which causes exocytosis of water channel vesicles. Mutations that disrupt membrane localization of aquaporin were described by Deen and colleagues in 1994 (Deen et al., 1994), and more recently Goji and colleagues described a mutation that disrupts the proper formation of the water pore (Goji et al., 1998).

Acquired diabetes insipidus can result from many causes. Brain injury can sever the infundibular stalk and prevent axonal transport of AVP (Vin-Christian & Arieff, 1993). Other cranial insults, including head injury-induced edema, encephalitis, Reye's Syndrome, subarachnoid hemorrhage, and anoxia also can cause diabetes insipidus (Reeder et al., 1986; Barzilay & Somekh, 1988). Very rare cases of diabetes insipidus arising from an autoimmune system disorder are also known (De Bellis et al., 1999).

AVP and AVP receptors control brain sodium and water levels, and alteration of their function in brain water and electrolyte balance can be severely deleterious (Raichle & Grubb, 1978; Doczi et al., 1982; Rosenberg et al., 1986; Faraci et al., 1990, 1994). As outlined later (see *Vasopressin's Potential Role in Choroid Plexus Function*), AVP may have an important role in the formation of edema after tissue injury.

Choroid Plexus Anatomy and Physiology

The main function of the choroid plexus epithelium is to provide the brain with a stable, nourishing milieu through the production of cerebral spinal fluid (CSF), and because of this function it has often been called the “kidney” for the brain (Spector & Johanson, 1989). The choroid plexus is a polarized epithelium with a basolateral surface that is in contact with the blood plasma through fenestrated capillaries, lateral surfaces that are connected to adjacent cells through tight junctions (one anatomical feature of the blood-CSF barrier), and an apical surface with microvilli (Spector & Johanson, 1989). The choroid plexuses are located in the third, fourth and lateral ventricles of the brain (Spector & Johanson, 1989). Normal choroid plexus epithelial cells have a light electron density of cytoplasm and a small perivascular space, but exposure to AVP has been shown to alter their cytoplasmic appearance from light to dark, enlarge their perivascular space and reduce their microvilli (Schultz et al., 1977; Liszczak et al., 1986; Johanson et al., 1999). These changes in morphology and appearance have been postulated to be due to a reduction in the secretion of CSF by the choroid plexus epithelial cells, and this hypothesis was recently bolstered when AVP treatment was found to reduce the efflux of Cl^- ions; an effect that could be reversed by the addition of a V_1 receptor antagonist (Johanson et al., 1999).

Brain water regulation plays an important role in neural brain function. Brain water is comprised of interstitial water (and its ionic components), intracellular water and the water contained in CSF. Generally, interstitial water and CSF have the same chemical composition. During fluctuations in plasma levels of nutrients and ions (osmolality), the

blood-brain and blood-CSF barriers function to maintain constant levels of those nutrients and ions in the brain (Cutler & Spertell, 1982). The choroid plexus, the *blood-CSF barrier*, actively controls the transport of ions and micronutrients (vitamin C, B₆, and folate) into the CSF. The cerebral capillaries, the *blood-brain barrier*, actively control the transport of larger and more readily used compounds (glucose, amino acids, lactate, and ribonucleotides) into the interstitial fluid. The production of CSF by the choroid plexus in humans is impressively high (~500ml/day) (Cutler & Spertell, 1982; Fishman, 1992). The majority of the water contained in CSF is removed by the arachnoid villi and drained into the venous sinuses (Fishman, 1992).

Levels of brain water and ions parallel changes that occur in the plasma (Fishman, 1992). When plasma osmolality is increased, the concentration of Na⁺, Cl⁻, and K⁺ in brain water are increased (Cserr et al., 1987; Fishman, 1992). Choroid epithelial cells take up Na⁺ ions from plasma and transport them into the CSF, resulting in the transport of negatively-charged ions (Cl⁻, bicarbonate) and the passive diffusion of water into the CSF (Cutler & Spertell, 1982). In addition, water diffuses freely across the cerebral capillary epithelium to balance brain water concentration. The signal processes mediating changes in CSF formation is not fully understood. AVP, as outlined below, may play a crucial role in choroid plexus functions with regard to brain water content and ion transport.

Vasopressin's Potential Role in Choroid Plexus Function

Vasopressin has been found at high levels in the CSF following certain stimuli including osmotic stimuli, hemorrhage, hypoxia, and intracranial hypertension (Gauvin et

al., 1977; Szczepanska-Sadowska et al., 1983; Faraci et al., 1994). The intraventricular administration of AVP increases CNS capillary permeability (Raichle & Grubb, 1978; Doczi et al., 1982; Rosenberg et al., 1986), and decreases CNS capillary blood flow (Faraci et al., 1990, 1994). There appears to be three sources of CSF AVP: the release of AVP from neurons of the hypothalamo-choroidal tract (Brownfield & Kozlowski, 1977), plasma AVP crossing the blood-brain barrier (Banks & Kastin, 1987; Banks et al., 1987; Zlokovic et al., 1992), or from the choroid plexus (Rudman & Chawla, 1976; Raichle & Grubb, 1978; Chodobski et al., 1997). Brownfield and Kozlowski, using an antibody to AVP were able to trace fine, varicose axonal fibers from the PVN that coursed through the subependymal layer to terminate in the choroid plexus (Brownfield & Kozlowski, 1977). At least three publications have described a blood-brain barrier AVP transport system (Banks & Kastin, 1987; Banks et al., 1987; Zlokovic et al., 1993). The choroid plexus appears to also be a source of AVP. As early as the late 1970s, researchers demonstrated that protein extracts of choroid plexus had antidiuretic activity (Rudman & Chawla, 1976; Raichle & Grubb, 1978). More recently, Chodobski showed the presence of AVP mRNA in the choroid plexus (Chodobski et al., 1997).

Vasopressin's actions on the blood-brain barrier may be significant in several clinical disorders. The presence of AVP in the CSF increases CNS capillary permeability (Raichle & Grubb, 1978; Doczi et al., 1982; Rosenberg et al., 1986), and decreases CNS capillary blood flow (Faraci et al., 1990, 1994). These two actions could potentially control the water content of the brain and be important in the management of edema. Head injury represents 26% of all accidental deaths that occur from injuries (Sosin et al.,

1989). Chesnut recently concluded the key treatment that improves a patient's eventual neurological status was the elimination of cerebral hypotension and hypoxia (Chesnut, 1997). Since edema results from hypotension and hypoxia, it is of paramount importance that we understand brain ion and water regulation. AVP and hypertonicity both cause a dramatic increase in the phosphorylation of an endothelial Na-K-Cl cotransporter protein causing an increase in its ion transport activity (Haas, 1994; O'Donnell et al., 1995). Intraventricular administration of a V_1 R antagonist blocks experimentally induced edema (Rosenberg et al., 1990). Interestingly, AVP and VR levels are higher in the CNS of Alzheimer's patients, although the reason for the increase is unknown (Korting et al., 1996; Labudova et al., 1998).

Chapter Two Experimental Rationale and Design

To develop a better understanding of vasopressin's role in brain electrolyte and water balance, we must first develop a more detailed understanding of the molecular mechanisms regulating AVP gene transcription. The measurement of mRNA levels is routinely used to monitor changes in gene expression, but there are limitations to this approach because of the delay between the time required for a stimulus received at the cell membrane to reach the cell's nucleus via second messenger systems, and the time between the conversion of heteronuclear RNA (hnRNA) to cytoplasmic mRNA (intron splicing, polyadenylation, 5' capping, and translocation of the mRNA to the cytoplasm). The splicing of hnRNA involves three steps (Sittler et al., 1986). One to two minutes after hnRNA is formed endonucleolytic cleavage occurs at the 5' splice site forming a lariat structure. One to two minutes later, endonucleolytic cleavage occurs at the 3' splice site

and the two ends of the exons are ligated together.

The splicing of introns from hnRNA is coupled to transcription, and excised introns are degraded within minutes (Bentley, 1999). Splicing factors that excise introns from nascent RNA transcripts have been localized to sites of active transcription within the nucleus called perichromatin fibrils (Spector, 1996; Misteli et al., 1997; Bentley, 1999). In addition, the carboxy-terminal domain (CTD) of RNA polymerase II, the enzyme responsible for the transcription of nascent RNA, has been found to bind essential splicing factors (McCracken et al., 1997). When the CTD of RNA polymerase II was removed, splicing was severely inhibited (McCracken et al., 1997). Thus, even though introns are spliced from the primary transcript, they can be detected and utilized as an indicator of transcriptional activity.

In situ hybridization with nucleic acid probes to intronic regions of a gene allows for the study of changes in transcription at the level of the single cell. In addition, investigators have previously demonstrated instances where physiological stimulation causes a rapid alteration in intronic levels, often on the order of minutes following the stimulus (Herman et al., 1991; Shoji et al., 1994). An ip injection of hypertonic saline, for example, increases AVP hnRNA in the SON above normal levels as soon as five minutes after administration (Ma & Aguilera, 1999), and hnRNA levels peaked by 30 minutes (Arima et al., 1999). To further develop the model of stimulus-secretion-transcription coupling (Sherman, 1996), I will examine the relationship between neuronal activation by hypertonic saline administration and transcription regulation of AVP. ***I hypothesize that presentation of hypertonic saline, compared to administration of an injection of***

normal saline, or no injection at all, will cause an increase in AVP hnRNA transcript levels of the vasopressin-synthesizing neurons of the supraoptic and paraventricular nuclei. Repeated alterations in plasma osmolality is known to cause an associated increase in neuronal activation of magnocellular neurons of the SON and PVN (Brimble & Dyball, 1977). *Since repeated administration of hypertonic saline is known to reliably increase neuronal activity, it is further hypothesized, based upon the stimulation-secretion-transcription model, that repeated injections of hypertonic saline will reactivate AVP gene transcription.*

In Chapter Two, changes in AVP transcription were evaluated by employing an *in situ* hybridization method using ^{32}P -labeled, random-primed DNA probes against AVP hnRNA. Two paradigms were used to investigate the relationship between ip injections of normal and hypertonic saline, and increases in the transcription of the vasopressin gene. A single-injection paradigm was used to evaluate the latency and duration of increased transcription due to osmotic stimulation. Animals were not injected, or injected with normal or hypertonic saline, and sacrificed fifteen or thirty minutes later. A final group was injected with hypertonic saline and sacrificed 180 minutes later. A double-injection paradigm was used to determine if AVP hnRNA levels undergo changes due to a second change in plasma osmolality. In the first group, animals were injected with normal saline followed 180 minutes later by a second injection of normal saline, and then sacrificed 30 minutes later (N-N group). In the second group, animals were injected with normal saline followed 180 minutes later by a second injection of hypertonic saline, and then sacrificed 30 minutes later (N-H group). Finally, in the third group animals were injected with

hypertonic saline followed 180 minutes later by a second injection of hypertonic saline, and then sacrificed 30 minutes later (H-H group).

Chapter Three Experimental Rationale and Design

To further understand vasopressin's role in controlling the intranuclear release of AVP within the SON and PVN, it is imperative to determine if $V_{1b}R$ gene regulation is altered by chronic osmotic stress. AVP is released by magnocellular neurons within the SON, and may bind to $V_{1b}R$ to initiate autoregulatory mechanisms (Ludwig, 1995). Recently, $V_{1b}R$ mRNA has been localized in the SON (Hurbin et al., 1998; Vaccari et al., 1998). ***I hypothesize that $V_{1b}R$ mRNA will be increased due to chronic osmotic stress in magnocellular neurons of the SON and PVN. This would help strengthen the theory that autoregulatory mechanisms are acting through $V_{1b}R$.***

The role AVP plays in controlling the secretion of CSF by the choroid plexus epithelium is important because certain disease states could be effectively treated by altering CSF secretion. Recently, AVP mRNA and $V_{1b}R$ mRNA have been localized within the choroid plexus. The formation of CSF by the choroid plexus is decreased due to exposure to increased plasma hyperosmolality and treatment with AVP (Schultz et al., 1977; Liszczak et al., 1986; Johanson et al., 1999). However, osmotic stimuli do not cause an increase in CSF levels of AVP (Wang et al., 1982; Sorensen & Hammer, 1985). Therefore, AVP and $V_{1b}R$ could be involved in the regulation of CSF formation by the choroid plexus during osmotic stress. ***I hypothesize that both proteins are involved in CSF formation during salt-loading, and therefore both genes will be transcriptionally enhanced resulting in increased levels of AVP and $V_{1b}R$ mRNA as compared to control***

animals.

In situ hybridization using riboprobes against the 3' end of AVP mRNA, and V_{1b}R mRNA, were utilized to detect changes in gene transcription in the SON and PVN of the hypothalamus, as well as in the choroid plexus. Animals were salt-loaded for one week, a chronic osmotic stressor that is known to increase plasma osmolality, decrease brain water, and cause a two-fold increase in the amount of AVP mRNA in the SON and PVN (Sueamaru et al., 1990).

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Chapter Two

Transcriptional Responses of the Vasopressin Gene to Acute and Repeated Acute Osmotic Stress

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Abstract

A radiolabeled probe complementary to the first intron (AVP1) of the rat arginine vasopressin (AVP) gene was used to detect changes in transcription of AVP heteronuclear RNA (hnRNA) in rat supraoptic nucleus neurons after hypertonic saline administration. Compared to animals given a single injection of normal saline, animals that received a single injection of hypertonic saline had increases in AVP1 after 15 and 30 minutes, with a return to baseline levels by 180 minutes. In a double injection paradigm, animals were given an injection of normal or hypertonic saline followed 180 minutes later by a second injection of normal or hypertonic saline and sacrificed 30 minutes later. When both injections were hypertonic saline (H-H) the levels of AVP1 were equivalent to the response seen after a single hypertonic saline injection. The levels of AVP1 after H-H treatment were also greater than the levels detected from animals given an injection of normal saline followed by a second injection of hypertonic saline (N-H). This is the first study utilizing an acute, repeated osmotic stress paradigm to show repeated exposures to hypertonic saline causes a robust increase in vasopressin gene transcription. Since a second hyperosmotic stimulus is known to increase neuronal firing rate and activity, our results suggest that a correlation exists with intracellular mechanisms regulating vasopressin gene transcription.

Introduction

Arginine vasopressin is a neuroendocrine hormone released into the plasma primarily by magnocellular neurons of the hypothalamo-neurohypophyseal system. The main physiological action of the hormone is the regulation of plasma osmolality, measured

as plasma $[Na^+]$ levels, through its effects on the distal tubule of the kidney. In 1947, Verney showed that an increase in plasma osmolality caused an increase in the plasma concentration of vasopressin within a matter of seconds (Verney, 1947). This effect can be reproduced through the administration of an intra peritoneal (ip) injection of hypertonic saline. An ip injection of hypertonic saline significantly elevates plasma $[Na^+]$ after 15 minutes (Dunn et al., 1973; Brimble & Dyball, 1977; Stricker & Verbalis, 1986; Shoji et al., 1994). In addition, an ip injection of hypertonic saline triggers a linearly related increase in plasma AVP (Dunn et al., 1973; Stricker & Verbalis, 1986). Although plasma $[Na^+]$ increases rapidly, the reduction of plasma $[Na^+]$ by the kidneys occurs much slower. Plasma $[Na^+]$ levels are decreasing 30 minutes after the injection of hypertonic saline, but are still elevated above normal levels after 180 minutes (Shoji et al., 1994; Wang, et al., 1997). The release of hormone, due to an increase in plasma osmolality, reduces hormone stores in the nerve terminal resulting in an increase in transcription of the vasopressin gene to replace those stores. This relationship is termed stimulus-secretion-transcription coupling (Sherman, 1996). In spite of the rapid secretion of hormone, AVP mRNA levels do not increase above normal levels until 120-180 minutes after osmotic stimulation, and remain elevated after 360 minutes (Herman et al., 1991; Shoji et al., 1994). Consequently, even though plasma levels of hormone are decreasing, AVP mRNA levels remain elevated, presumably to replenish AVP hormone stores in the nerve terminal.

Investigators have firmly established that hormone release due to increases in plasma osmolality are related to increased neuronal activity. In 1977, Brimble and Dyball investigated how repeated injections of hypertonic saline increased magnocellular neuronal

activity (Brimble & Dyball, 1977). A second injection of hypertonic saline increased the firing rate and duration of activity of vasopressinergic neurons in the SON even higher than the levels seen after the first injection (Brimble & Dyball, 1977). Osmotic stimuli also induce the local release of AVP within the SON, which is theorized to be autoregulatory (Landgraf & Ludwig, 1991), and may have an inhibitory action on highly active SON neurons (Gouzenes et al., 1998). If AVP hnRNA levels are elevated after two hypertonic saline injections, results are in line with the work of Brimble and Dyball, and strengthen the link between neuronal firing patterns and transcriptional regulation of AVP (Brimble & Dyball, 1977). The effects of a second injection, administered 180 minutes after the first injection, were examined because plasma osmolality is still elevated at that time and we wanted to assess the effects of a second injection when AVP mRNA levels are beginning to increase due to the first injection. The animals were then sacrificed 30 minutes after the second injection to allow comparison to the single injected animals.

Materials and methods

Animal procedures

Adult male, Long-Evans rats weighing 175-225 g were housed in groups of 2-3 per cage with a 14-10 h light-dark cycle and food and water available *ad libitum*. To diminish the stress response from in-house transport, the rats were moved from the university's animal facility to the laboratory the night before experimental treatment. All methods were performed according to University Laboratory Animal Review Board guidelines. Prior to treatment, food and water were removed from the cage. Two treatment paradigms were utilized; single injections and double injections. For single

injection studies, rats were not injected, or received an ip injection of either normal saline (0.9% NaCl) or 1.5 M hypertonic saline at a concentration of 0.018 ml/g. Rats were sacrificed 15, 30, or 180 minutes post-injection ($n = 3-5$). Rats were not injected with normal saline and sacrificed after 180 minutes since there were no increases in AVP1 detected in the NS15' and NS30' groups. For double-injection studies, the rats were injected with normal or hypertonic saline, followed 180 minutes later by a second injection of normal or hypertonic saline, and were sacrificed 30 minutes later ($n = 4-5$). Brains were removed, blocked to isolate the hypothalamus and stored in liquid nitrogen. Frozen blocks were sectioned in the coronal plane (14 μ m thick) using a Bright-Hacker cryostat. The sections were thaw-mounted on Chrome-Alum gelatin-coated glass slides and were post-fixed in 4% paraformaldehyde at a ratio of 1:1 with phosphate buffered saline (PBS) for 5 minutes, equilibrated in 1X PBS for 5 minutes, rinsed in distilled water for 2 minutes, dehydrated in increasing concentrations of ethyl alcohol for 2 minutes each, cleared in chloroform for 5 minutes, and rinsed in 100% ethanol for 3 minutes. The slides were stored in a vacuum desiccator.

In situ hybridization

Using *MacVector* 4.5 sequence analysis software (Oxford Molecular Group, Beaverton, OR), primers were designed for the polymerase chain reaction (PCR) to amplify a 502 nucleotide fragment [14,361-14,862 (Schmale et al., 1983)] from intron 1 of the vasopressin gene. Rat cerebellar genomic DNA was used as template. The PCR reaction mix (Perkin Elmer GeneAmp PCR Reagent Kit) included a final concentration of 1X PCR buffer (2 mM $MgCl_2$), 200 μ M each dNTP, 1.25 U AmpliTaq DNA polymerase,

0.47 μ M forward primer, 0.47 μ M backward primer, 2.0 μ g cerebellar genomic DNA, and enough sterile water to bring the final volume to 50 μ l. The PCR reaction conditions were carried out in a Perkin Elmer GeneAmp PCR System 9600 thermocycler. The mixture was "hot started" by overlaying with Ampliwax PCR Gem 50s (Perkin Elmer). The cycle started with a 1 minute Ampliwax melt step at 95°C, followed by 30 cycles of 96°C for 1 minute/55°C for 1.33 minute/72°C for 2 minutes, finishing with an extra extension at 72°C for 2 minutes. The PCR products were analyzed on a 2% TBE agarose gel and bands corresponding to the correct size were excised and purified using a Promega Wizard PCR Preps DNA Purification System Kit.

The purified PCR products were ligated into Promega's pGEM-T vector and JM109 cells were transformed with the plasmid construct. Transformed cells were streaked on LB plates precoated with ampicillin (100 μ g), X-gal (0.8 mg), and IPTG (0.2 mg) and grown overnight at 37°C. White colonies with potential inserts were selected and grown overnight at 37°C in 2.5 ml of LB broth and ampicillin (30 μ g/ml). Plasmid DNA was purified using Promega's Wizard *Plus* Minipreps DNA Purification System Kit. To insure the correct insert was amplified, the insert sequence was checked by dideoxy-fluorescent sequencing (Biomedical Instrumentation Center, USUHS).

The inserts were excised from the plasmid DNA using the restriction enzymes *Sac* II and *Spe* I (Promega Corporation) overnight at 37°C. The fragments were gel purified on 2% TAE agarose gels, and based on size the correct bands were excised and purified from gel slices using Promega's Wizard PCR Preps DNA Purification System Kit. The gel-purified DNA was used as template in ³²P-dCTP (Amersham) random priming

reactions using Gibco BRL's RadPrime DNA Labeling System. The labeled probe was purified using G-50 Sephadex spin columns (Boehringer-Mannheim), and counted using a Beckman scintillation counter.

Brain sections on slides were saturated with pre-hybridization buffer as previously described for 60 minutes at 43°C (McCabe & Pfaff, 1989). After pre-hybridization, the brain sections were saturated with hybridization buffer as previously described, containing a concentration of 150,000 to 500,000 counts per section of probe, overnight at 43°C (McCabe & Pfaff, 1989). The slides were washed in 2X SSC, 1X SSC, 0.2X SSC, & 0.1X SSC for 60 minutes each in a 43°C agitating water bath, followed by one minute washes in ethanol and ammonium acetate for one minute each (McCabe & Pfaff, 1989). The slides were dried under vacuum, exposed to Biomax film (Eastman Kodak) for ~65 hours, dipped in NTB3 autoradiography emulsion (Kodak), and exposed for two to three weeks in desiccating boxes at 4°C.

Autoradiograms were developed in D-19 (Kodak) for 2 minutes at 16°C, rinsed briefly in water at 19°C, fixed for 10 minutes in fixer (Kodak) at 19°C, and rinsed in water. The slides were counter-stained in cresyl violet (0.1 g/100 ml), rinsed twice in water, once in 70% ethanol, once in 95% ethanol, twice in 100% ethanol, cleared in Hemo-De (Fisher Scientific) and mounted in DPX (Fluka Chemika).

Analysis and quantification

Optical density (OD) measures of film background, background-labeled neuropil, and individual supraoptic nuclei were performed using a NEC color CCD camera (model number NX18AS) and a custom software program on a Next Dimension computer. To

standardize measurements of autoradiographic grain densities across films and across experiments, film OD and neuropil OD of a representative film were first assessed at different levels of light intensity. The values were plotted to determine the range in which the two values had the greatest difference. The range was determined to be standardized when the neuropil OD was between 90 and 210 units (on a grey scale of 256 units). To eliminate potential complications arising from instances of film saturation, defined as the overexposure of the film due to excessive radioactivity as a function of length of exposure, a criterion was also established to only analyze films where the average neuropil OD was less than 210 grey scale units. Following standardization of each autoradiogram, three OD values from each SON were averaged and this value was subtracted from the average OD of the neuropil from the same tissue sample. The resulting OD values for several samples of SON from each animal, encompassing several different hybridizations, were averaged. One-way analysis of variance and the Student-Newman-Keuls test (Sigma Stat software) were used to evaluate the means and variances for each treatment group.

Results

Single injection paradigm

The optical density measures of AVP1 in the SON of rats that received no injection (NI group), or injections of normal or hypertonic saline are summarized in Figure 1. The mean optical density of AVP1 from SON samples of rats that received an injection of normal saline and were sacrificed fifteen (NS15'=127%) or thirty (NS30'=163%) minutes later were not significantly greater than levels detected in the NI group (see also Figure 2, A and B). When hypertonic saline was injected, a significant increase ($p < 0.05$)

Figure 1. Osmotic regulation of vasopressin intron 1 levels in the SON due to a single injection or double injection of normal (NS) and/or hypertonic saline (HS). Single injection rats were sacrificed 15, 30, or 180 minutes post-injection. Significant increases ($p < 0.05$) were seen at all hypertonic saline time points (HS15', $n=4$; HS30', $n=4$; & HS180', $n=5$) when compared to the non-injected group (NI, $n=5$). However, only the HS30' group was significantly different ($p < 0.05$) from the normal saline groups (NS15', $n=3$ & NS30', $n=3$). Rats that were injected with normal saline followed 180 minutes later by a second injection of normal saline, and sacrificed 30 minutes later (N-N, $n=4$) were significantly different ($p < 0.05$) from the HS30', N-H, and H-H groups. When the first injection was normal saline and the second injection was hypertonic saline (N-H, $n=4$), or when the first injection was hypertonic saline and the second injection was hypertonic saline (H-H, $n=5$), optical density measures were significantly greater ($p < 0.05$) than the NI and NS15' groups. Like the HS30' group, the H-H group was also significantly different ($p < 0.05$) from the normal saline groups (NS15' & NS30'). However, the HS30' and H-H groups were not significantly different from each other.

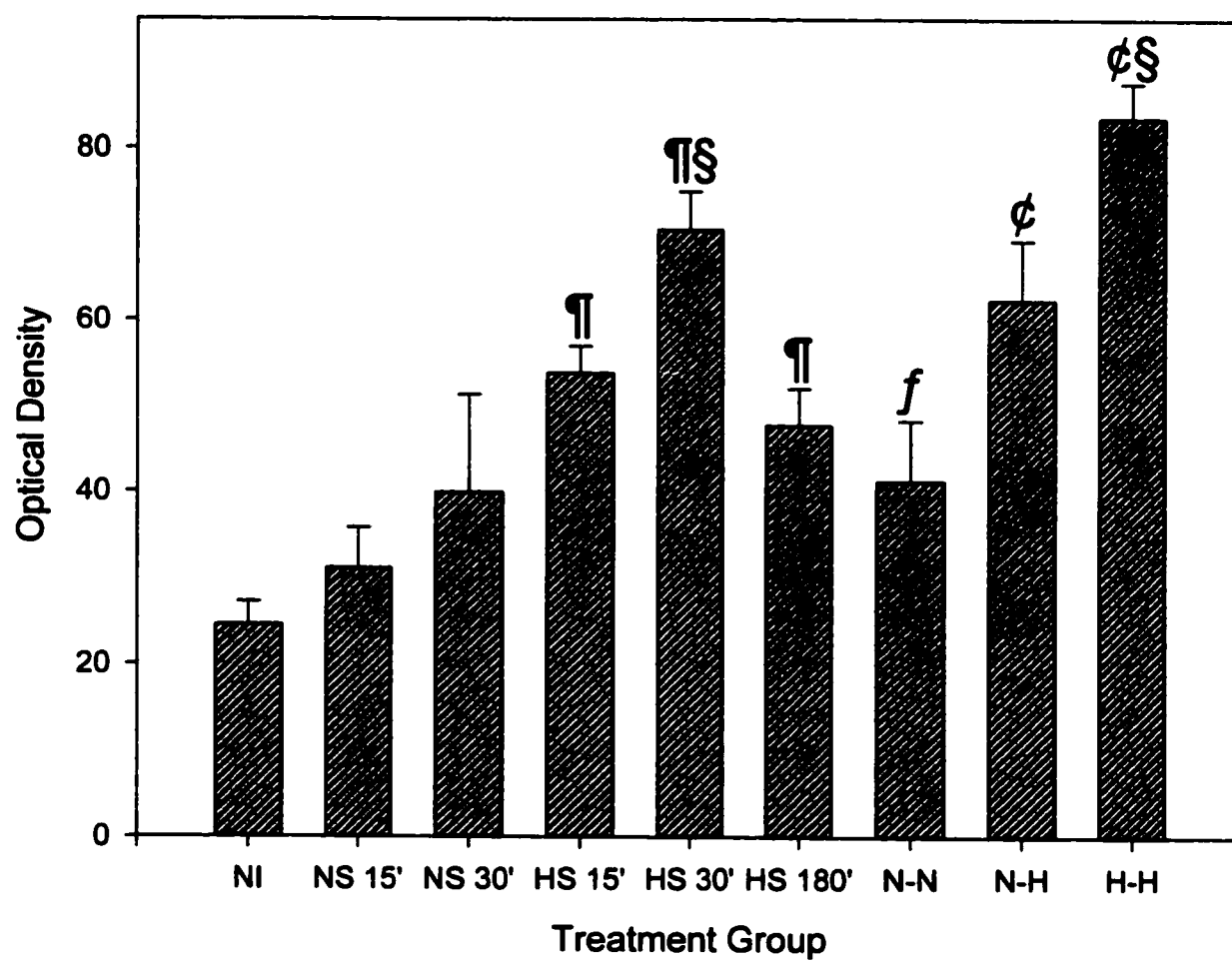
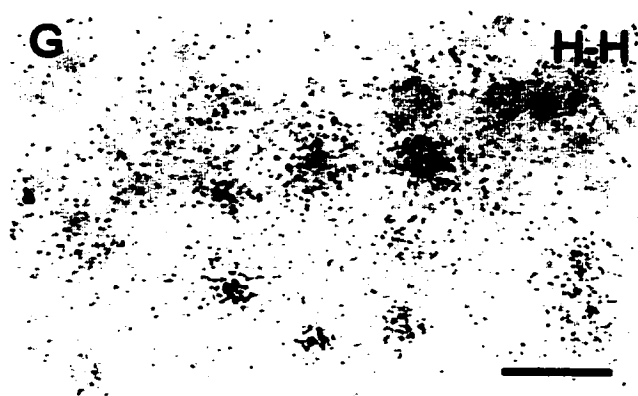
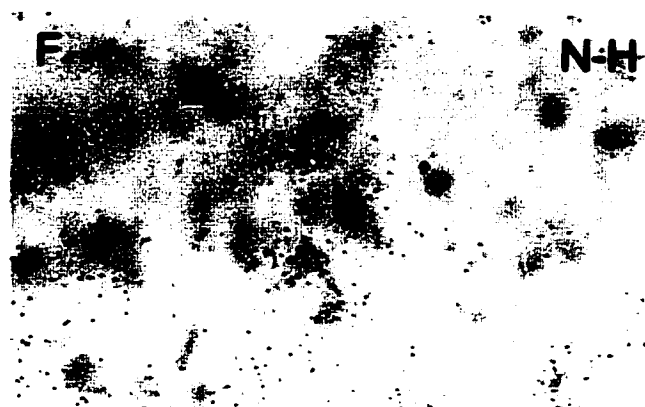
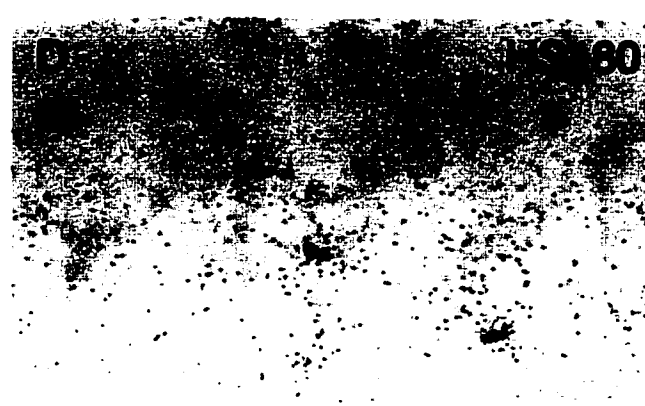
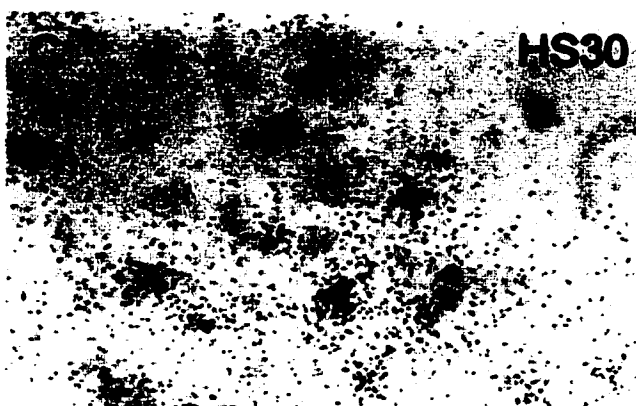
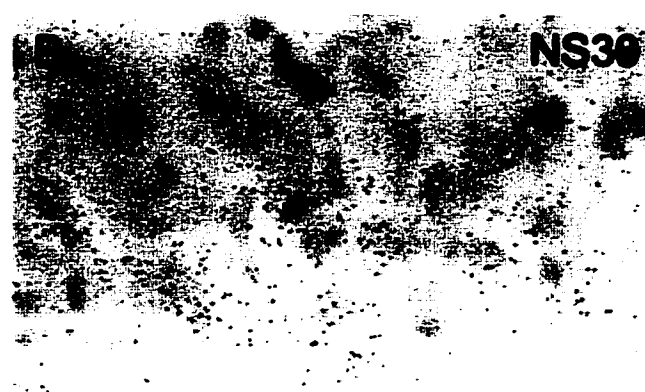
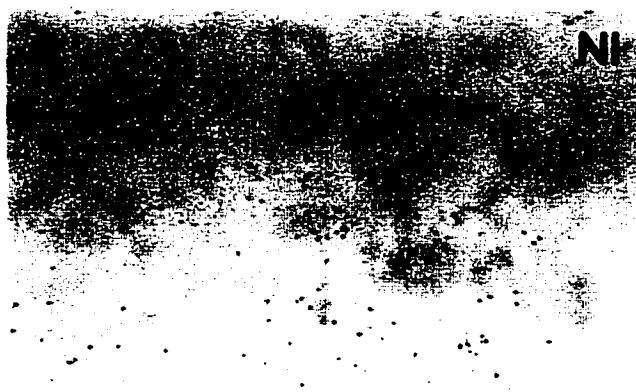


Figure 2. Bright-field micrographs of vasopressin intron 1 (AVP1) hnRNA localized in rat supraoptic nucleus (SON), visualized by *in situ* hybridization histochemistry. A 502 nucleotide fragment from AVP1 was used to generate random primed ³²P-labeled DNA probe. The slides were counter-stained with cresyl violet. The animal treatment groups were non-injected (NI: A), injected with normal saline and sacrificed 30 minutes later (NS30: B), injected with hypertonic saline and sacrificed 30 minutes later (HS30: C), injected with hypertonic saline and sacrificed 180 minutes later (HS180: D), injected with normal saline followed 180 minutes later by a second injection of normal saline, and sacrificed 30 minutes later (N-N: E), injected with normal saline followed 180 minutes later by an injection of hypertonic saline, and sacrificed 30 minutes later (N-H: F), and injected with hypertonic saline followed 180 minutes later by a second injection of hypertonic saline, and sacrificed 30 minutes later (H-H: G). The same magnification was used for all pictures and the bar in G equals 25µm.



of vasopressin hnRNA levels in the SON was detected after fifteen (HS15'=219%), thirty (HS30'=288%) and 180 (HS180'=194%) minutes, compared to levels measured in the NI group. However, the AVP1 levels in the HS15' group were not significantly different from levels detected in the HS30' group or the normal saline groups. Only the AVP1 levels in the HS30' group were significantly higher ($p<0.05$) than levels detected in the normal saline groups (also see Figure 2 B, C and E). The levels of AVP1 in the SON 180 minutes after an injection of hypertonic saline (HS180' group) were significantly lower ($p<0.05$) than levels detected in the HS30' group, but were not significantly different from levels detected in the normal saline groups (see Figure 2 B, C, D and E).

Double injection paradigm

The mean optical density of AVP1 in the SON of rats given an injection of normal saline, followed 180 minutes later by another injection of normal saline, and sacrificed 30 minutes later (N-N group=168%), was not significantly more than the NI group (see Figure 1 and Figure 2, A and E). When the second injection was hypertonic saline (N-H group), AVP1 levels in the SON were significantly higher (254%; $p<0.05$) than levels observed in the NI group (see Figure 2, A and F). However, the levels of AVP1 detected in the N-H group were only significantly less ($p<0.05$) than AVP hnRNA levels in animals that received two hypertonic saline injections. Levels of AVP1 in the H-H group were significantly higher (341%; $p<0.05$) than every other group except the HS30' group (see Figures 1 and 2).

Discussion

The administration of a hypertonic stimulus is a potent releaser of AVP from the

posterior pituitary. Transcriptional and translational pathways are activated to restore the level of hormone in the nerve terminal. Levels of vasopressin intron 1 RNA in the SON were elevated fifteen minutes after a single injection of hypertonic saline, further elevated 30 minutes post-injection, and remained high for at least 180 minutes post-injection. Our results are in accordance with previously reported observations (Herman et al., 1991; Shoji et al., 1994; Ma et al., 1999).

Previous reports have utilized either non-injection or normal saline injections as comparisons to experimental treatment. This may be the first study to directly compare the effects of non-injection and normal saline injection on AVP hnRNA levels in rats. When a needle was inserted into the abdominal cavity but no injection made, there were no changes in AVP hnRNA (Shoji et al., 1994). In the present study we found infusing normal saline lead to a slight increase in the amount of AVP hnRNA (NS15' and NS30'), compared to levels seen in a non-injected animal (NI), but this difference was not significant. AVP1 levels of the HS15' were significantly different than the NI and HS30' groups, but were not significantly different from the normal saline groups (NS15', NS30', & N-N). This finding differs from a recent report that found a significant increase in AVP hnRNA in the SON 10 minutes after an ip injection of hypertonic saline compared to levels seen after an ip injection of normal saline (Arima et al., 1999). The amount of AVP1 peaked at HS30' confirming previous reports using Sprague-Dawley rats (Herman et al., 1991; Arima et al., 1999; Ma et al., 1999). The AVP1 levels at HS180' were significantly less than the HS30' group, and decreased to a point where they were not significantly different from the normal saline groups (NS15', NS30', & N-N). Although

AVP1 levels at HS180' were returning to normal levels, the possibility exists that they could exhibit a second increase as others have reported (Ma & Aguilera, 1999).

We define our double-injection paradigm as acute since the second injection was administered before the transcriptional response from the first injection had fully subsided (Lafarga et al., 1998). The injection of normal saline followed by an injection of hypertonic saline caused AVP1 levels to increase to a position intermediate between the normal saline group levels (NS15', NS30', & N-N) and the hypertonic saline levels (HS30' & H-H). These results suggest normal saline administration, perhaps acting as a pre-load of body fluid volume, diminished the osmotic impact of the subsequent injection of hypertonic saline. Normal saline (Pendlebury et al., 1992) and water (Brimble & Dyball, 1977) administration has been shown to lead to diminished neuronal activity for at least 60 minutes.

Similar to treatments with a single injection of hypertonic saline, double injections of hypertonic saline (H-H) significantly raised AVP hnRNA levels. Just as Brimble and Dyball found that a second injection of hypertonic saline increased the firing rate of magnocellular neurons (Brimble & Dyball, 1977), we found that a second injection of hypertonic saline (H-H) was able to increase AVP hnRNA levels to the same extent than after a single injection. We did not observe, however, an even greater enhancement of hnRNA levels, which would have paralleled the electrophysiological findings of Brimble and Dyball. Our procedures differed from the latter work, however, and this may be important. Brimble and Dyball used female rats that were pregnant and anesthetized. In addition, the second infusion was administered within 60 minutes of the first injection,

while the duration between injections in our study was 180 minutes. The “additive” effect on supraoptic nucleus neuron electrical activity may have been more akin to a dose response effect, since the hyperosmotic state from the first infusion would not have subsided (Wang, et al., 1997). Finding a similar elevation of AVP hnRNA after one and two hypertonic saline injections may be relevant to the findings of Lafarga and colleagues (Lafarga et al., 1998). These investigators provided ultrastructural observations suggesting that a single injection of hypertonic saline caused inactivation of transcriptional activity. Two hours after hypertonic saline administration, the cell nuclei of supraoptic nucleus neurons appeared shriveled, and contained enlarged clusters of interchromatin granules and condensed chromatin. Explants were tested for ^3H -uridine incorporation, and found to be significantly lower in hyperosmotic rats than levels observed in non-injected animals. In the present study, we have found what Lafarga describes as one of the exceptions to transcriptional inactivation. Following stimulation with a second injection of hypertonic saline, AVP hnRNA levels appeared to not exhibit diminished transcriptional activity. It has been hypothesized that the firing rate of these neurons is coupled to the transcription of the AVP gene either directly, or through the depletion of hormone stores in the nerve terminal (Sherman, 1996). Electrophysiological studies, coupled with measurement of transcription, are needed to directly relate neuronal excitation with transcriptional changes.

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Chapter Three

Salt-loading Increases Vasopressin and Vasopressin 1b Receptor mRNA in the Hypothalamus and Choroid Plexus

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Abstract

The choroid plexus plays a pivotal role in the production of cerebrospinal fluid (CSF). Messenger RNA (mRNA) transcripts encoding arginine vasopressin (AVP) and the vasopressin 1b receptor ($V_{1b}R$) are found in various structures of the central nervous system, including the choroid plexus. The present study measured AVP and $V_{1b}R$ mRNA production in response to plasma hyperosmolality, where rats were given tap water or 2% saline as drinking solution for 7 days. Compared to rats maintained on water, salt-drinking rats had increased levels of AVP and $V_{1b}R$ mRNAs in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus, as well as in the choroid plexus. Brain water content decreased in rats that were salt-loaded. The increase in AVP and $V_{1b}R$ mRNAs in the SON and PVN as a result of plasma hyperosmolality may potentially be involved in the intranuclear release of AVP that autoregulates the activity of hypothalamic magnocellular neurons. The increase of AVP and $V_{1b}R$ mRNAs in the choroid plexus further shows the involvement of AVP in the regulation of brain water content and cerebral edema.

Introduction

Arginine vasopressin is best known for its antidiuretic functions, regulating water reabsorption by the distal tubule of the kidney. AVP is also present at high levels in the cerebrospinal fluid (CSF) following certain stimuli, including plasma hyperosmolality, hemorrhage, hypoxia, and intracranial hypertension (Szczepanska-Sadowska et al., 1983; Faraci et al., 1994). The origin of CSF AVP may arise from three sources: transport into the CSF from peripheral blood (Banks et al., 1987; Tanabe et al., 1999), central release

from hypothalamic neurons (Brownfield & Kozlowski, 1977), and *in situ* synthesis and release into the CSF from epithelial cells of the choroid plexus (Rudman & Chawla, 1976; Chodowski et al., 1997, 1998a). The significance of CSF AVP is not fully understood, but may be related to regulation of brain water content (Rodriguez, 1976; Rudman & Chawla, 1976; Dóczy, 1993). Increased ventricular levels of AVP, where levels are normally an order of magnitude higher in CSF than in plasma (Szczepanska-Sadowska et al., 1983), increase brain water content (Dóczy et al., 1982). The intraventricular administration of AVP increases capillary permeability (Raichle & Grubb, 1978; Dóczy et al., 1982; Rosenberg et al., 1986), and decreases capillary blood flow (Faraci et al., 1990, 1994). Faraci and colleagues (Faraci et al., 1990) have shown that intracerebroventricular injection of AVP reduces choroid plexus CSF production by 35%. During hypoxia (ventilation with 8% O₂) or increased intracranial pressure (infusion of artificial CSF) there was a minimal increase in plasma AVP levels, yet a significant decrease in blood flow to the choroid plexus. A vasopressin V₁-antagonist was able to block the decrease in blood flow (Faraci et al., 1994). AVP may also play a role in cerebral edema (Faraci et al., 1990, 1994). Brain injury-induced edema is substantially reduced by the administration of V₁ receptor antagonists (Rosenberg et al., 1990; Nagao et al., 1994; Ikeda et al., 1997; László et al., 1999).

Vasopressin receptors form a distinct subgroup of the G protein-coupled, seven putative transmembrane domain receptor family (Burbach et al., 1995). V_{1a}R mRNA is localized throughout the neuraxis (cortical areas, hippocampus, hypothalamus, and brainstem nuclei), the circumventricular organs of the brain (subfornical organ and area

postrema), the cerebral blood vessels, and the choroid plexus (Ostrowski et al., 1992, 1994; Szot et al., 1994; Hurbin et al., 1998). Although less widespread, V_{1b} R mRNA is localized in the dentate gyrus, hippocampus, hypothalamus, anterior pituitary, and the choroid plexus (Burbach et al., 1995; Lolait et al., 1995; Hurbin et al., 1998). Originally, V_2 R mRNA was not detected in the central nervous system (Ostrowski et al., 1992), but others recently localized V_2 R mRNA in the cerebellum (Kato et al., 1995). Interestingly, V_2 R mRNA was detected in the choroid plexus of newborn rats, but was not detectable in the adult (Kato et al., 1995).

In this study, we measured levels of AVP and V_{1b} R mRNAs as a function of increased plasma osmolality. Hyperosmolality stimulates the release of AVP from neurohypophyseal axon terminals and is associated with increased AVP mRNA levels. AVP is also released locally from dendritic processes of AVP neurons, possibly for autoregulation of neuronal activity (Ludwig, 1998). Recently, V_{1b} R mRNA was localized in the SON (Hurbin et al., 1998; Vaccari et al., 1998), and autoregulatory processes in SON neurons may act through V_{1b} receptors (Dayanithi et al., 2000).

Materials and Methods

Animal procedures

Sixteen adult male, Long-Evans rats (200-225g) purchased from Charles River Labs were housed in groups of 2-3 per cage with a 14-10 h light-dark cycle and food and tap water available *ad libitum*. All methods were performed according to University Laboratory Animal Review Board guidelines. When the experiment commenced, one-half of the rats were provided with 2% saline as their sole drinking solution for seven days. To

diminish any potential stress from the novelty of in-house transport, the rats were moved from the university's animal facility to the laboratory the night before they were sacrificed. The animals were given Ketaset (80 mg/kg) and Rompun (13 mg/kg) ip, and when unresponsive to paw pinch, decapitated by guillotine. The brains were removed, sectioned with a sterile razor to isolate a region rostral to the hypothalamus and caudal to the hippocampus, and stored in liquid nitrogen (McCabe & Pfaff, 1989). Frozen blocks were sliced in the coronal plane (14 μ m thick) using a Bright-Hacker cryostat. The sections were thaw-mounted on Chrome-Alum gelatin-coated glass slides and were post-fixed and washed in solutions made with DEPC-treated water. Briefly, the slides were post-fixed in 4% paraformaldehyde at a ratio of 1:1 with phosphate buffered saline (PBS) for 5 minutes, equilibrated in 1X PBS for 5 minutes, rinsed in distilled water for 2 minutes, dehydrated in increasing concentrations of ethyl alcohol (70/95/100%) for 2 minutes each, cleared in chloroform for 5 minutes, and rinsed in 100% ethanol for 3 minutes. The slides were stored in a vacuum desiccator.

The tissue blocks obtained above for *in situ* hybridization were analyzed in conjunction with blood chemistry and brain water content. At the time of decapitation, trunk blood was collected, centrifuged at 3,000 X g for 10 minutes, and stored for 2-4 hours in a refrigerator (4°C) until it could be analyzed for osmolality and electrolytes (Na^+ , Cl^- , K^+) by the Clinical Pathology Laboratory, USUHS (Kodak Ektachem 700 Analyzer). Brain water content was assessed using the method described by Kagawa (Kagawa et al., 1996). Samples of the brain not used for *in situ* hybridization (the brain rostral to the preoptic area, caudal to the interpeduncular fossa, and temporal cortex lateral to the

diencephalon) from each animal were weighed to obtain their wet weight (WW). The samples were then dried for 2-4 days at 42°C in an oven, and weighed again to obtain their dry weight (DW). Brain water content is described as a percentage defined by the equation below.

$$\text{Brain Water Content} = \left[\frac{\text{WW}-\text{DW}}{\text{WW}} \right] \times 100$$

In situ hybridization

Using *MacVector* 4.5 sequence analysis software (Oxford Molecular Group, Beaverton, OR), primers were designed for the polymerase chain reaction (PCR) to amplify a 158 nucleotide fragment (13,282-13,440) from exon 3 of the vasopressin gene (Schmitz et al., 1991), and a 249 nucleotide fragment (1,192-1,440) from the 3' region of the V_{1b} receptor (Saito et al., 1995). The PCR reaction mix (Perkin Elmer GeneAmp PCR Reagent Kit) included a final concentration of 1X PCR buffer (2 mM MgCl₂), 200µM each dNTP, 1.25 U AmpliTaq DNA Polymerase, 0.47 µM forward primer, 0.47 µM backward primer, 2.0 µg cerebellar genomic DNA, and enough sterile water to bring the final volume to 50 µl. The PCR reaction conditions were carried out in a Perkin Elmer GeneAmp PCR System 9600 thermocycler. The reactions were "hot started" by overlaying with Ampliwax PCR Gem 50s (Perkin Elmer). The cycle started with a 1 minute Ampliwax melt step at 95°C, followed by 30 cycles of 96°C for 1 minute/55°C for 1.33 minute/72°C for 2 minutes, finishing with an extra extension at 72°C for 2 minutes. The PCR products were analyzed on a 2% TBE agarose gel and bands corresponding to the correct size were excised and purified using a Promega Wizard PCR Preps DNA Purification System Kit.

The purified PCR products were ligated into Promega's pGEM-T vector and JM109 cells were transformed with the plasmid construct. Plasmid DNA was purified using Promega's Wizard *Plus* Minipreps DNA Purification System Kit. To insure the correct insert was amplified, the insert sequence was checked by dideoxy-fluorescent sequencing (Biomedical Instrumentation Center, USUHS).

The orientation of the insert determined from sequencing was used to select the appropriate restriction enzyme to linearize the plasmid DNA for use in making run-off anti-sense transcripts described below. The plasmid DNA was linearized using the restriction enzymes *Sac* II and *Nco* I (Promega Corporation) for one hour at 37°C for the AVP mRNA probe and the V_{1b} receptor probe, respectively. Riboprobes were transcribed using T₇ or SP6 polymerase per vendor's instructions (Promega, Madison, WI). The reaction mixtures containing labeled riboprobe, in addition to a reaction mixture with labeled Century marker (Ambion), were separated on a denaturing 8% acrylamide gel. The labeled riboprobes were purified from gel slices using Ambion's RNA extraction buffer overnight at 37°C.

Slides containing brain sections were pre-hybridized for 60 minutes at 43°C, and then hybridized with 100,000 to 300,000 counts per section of probe overnight at 43°C (McCabe & Pfaff, 1989). The slides were then washed in 4XSSC/5mM DTT at room temperature for 5 minutes to remove excess probe before being washed three times in the same solution for 20 minutes each at room temperature. The slides were then washed twice in RNase buffer for 30 minutes at 37°C while being agitated, and RNase A added to the second wash to a final concentration of 1.25 µg/ml. The slides were then washed

twice in 2XSSC for 10 minutes at room temperature. Finally, the slides were washed in 0.1XSSC for 30 minutes at room temperature, followed by precipitation and dehydration in increasing concentrations of ethanol to decreasing concentrations of ammonium acetate for one minute each. The slides were dried under vacuum, exposed to Biomax film (Eastman Kodak) for ~65 hours, dipped in NTB3 autoradiography emulsion (Kodak), and exposed for two to four weeks in desiccating boxes at 4°C. Sections hybridized with sense riboprobes had no specific hybridization signal (data not shown).

Autoradiograms were developed in D-19 (Kodak) for 2 minutes at 16°C, rinsed briefly in water at 19°C, fixed for 10 minutes in fixer (Kodak) at 19°C, and rinsed in water. The slides were counter-stained in cresyl violet (0.1 g/100 ml), rinsed twice in water, once in 70% ethanol, once in 95% ethanol, twice in 100% ethanol, cleared in Hemo-De (Fisher Scientific) and mounted in DPX (Fluka Chemika).

Analysis and quantification

Optical density (OD) measures of film background, background-labeled neuropil, and individual choroid plexuses, SONs, or PVNs were performed using a NEC color CCD camera (model number NX18AS) and a custom software program on a Next Dimension computer. To standardize measurements of autoradiographic grain densities across films and across experiments, film OD and neuropil OD of a representative film were first assessed at different levels of light intensity. The values were plotted to determine the range in which the two values had the greatest difference. The range was determined to be standardized when the neuropil OD was between 90 and 210 units (on a grey scale of 256 units). To eliminate potential complications arising from instances of film saturation,

defined as the overexposure of the film due to excessive radioactivity as a function of length of exposure, a criterion was also established to only analyze films where the average neuropil OD was less than 210 grey scale units. Following standardization of each autoradiogram, three OD values from each SON were averaged and this value was subtracted from the average OD of the neuropil from the same tissue sample. The resulting OD values for several samples of SON from each animal, encompassing several different hybridizations, were averaged. One-way analysis of variance and the Student-Newman-Keuls test (Sigma Stat software) were used to evaluate the means and variances for each treatment group.

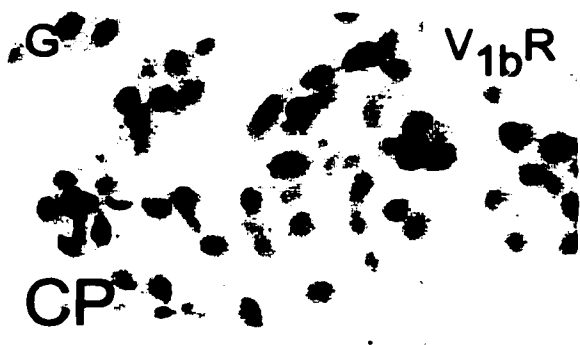
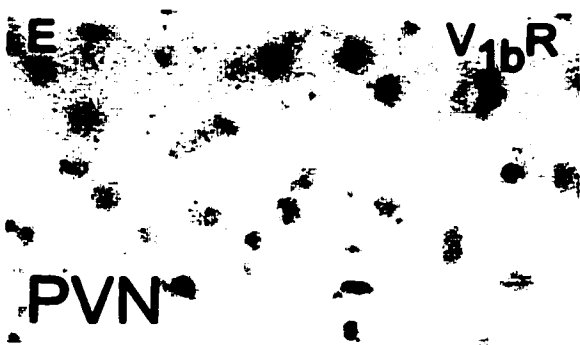
Results

Animals that were salt-loaded had significant increases ($p < 0.05$) in plasma osmolality and sodium concentration. The mean plasma osmolality increased significantly from 263.4 mOsmol/Kg in the control group to 292.1 mOsmol/Kg in the salt-loaded group, and mean plasma sodium increased from 135.0 mEq/l in the control group to 148.8 mEq/l in the salt-loaded group. The mean brain water content in the control group was 78.90%, and decreased significantly ($p < 0.05$) to 77.85% in the salt-loaded group.

Salt-loaded animals had increased levels of AVP mRNA in the supraoptic nucleus (SON), paraventricular nucleus (PVN), and the choroid plexus (Figures 1 & 2). Compared to the control group, salt-loaded rats had significant ($p < 0.05$) increases in AVP mRNA of 170% and 203% in magnocellular neurons of the SON and PVN, respectively. In the choroid plexus of salt-loaded rats AVP mRNA increased 423% compared to control animals ($p < 0.05$). Also, salt-loaded animals had a greater number of epithelial

Figure 1. Bright-field photomicrographs of arginine vasopressin (AVP) mRNA and vasopressin 1b receptor (V_{1b}R) mRNA localized in rat supraoptic nucleus (SON), paraventricular nucleus (PVN), and choroid plexus (CP) epithelium. *In situ* hybridization was performed on cryostat sections of brain tissue containing SON, PVN, and CP. A 158 nucleotide fragment from exon 3 of the AVP gene (A & B), and a 249 nucleotide fragment from the 3' region of the V_{1b}R gene (C-H), were used to generate ³²P-labeled riboprobes. Tissue samples from control animals (A, C, E & G), and animals salt-loaded for 7 days (B, D, F & H), were hybridized overnight (McCabe and Pfaff, 1989). Developed slides were counter-stained with cresyl violet. The number of V_{1b}R transcripts in magnocellular neurons of the SON and PVN increased due to salt-loading. Both AVP and V_{1b}R mRNA species increased in the epithelial cells of the choroid plexuses of salt-loaded animals. All photomicrographs were taken at the same magnification and the bar in H equals 25 μ m.

Control



Salt-Loaded

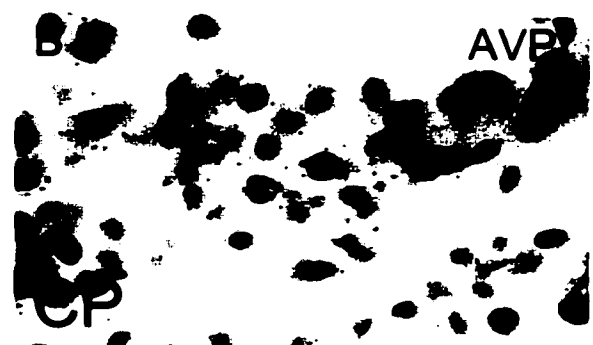
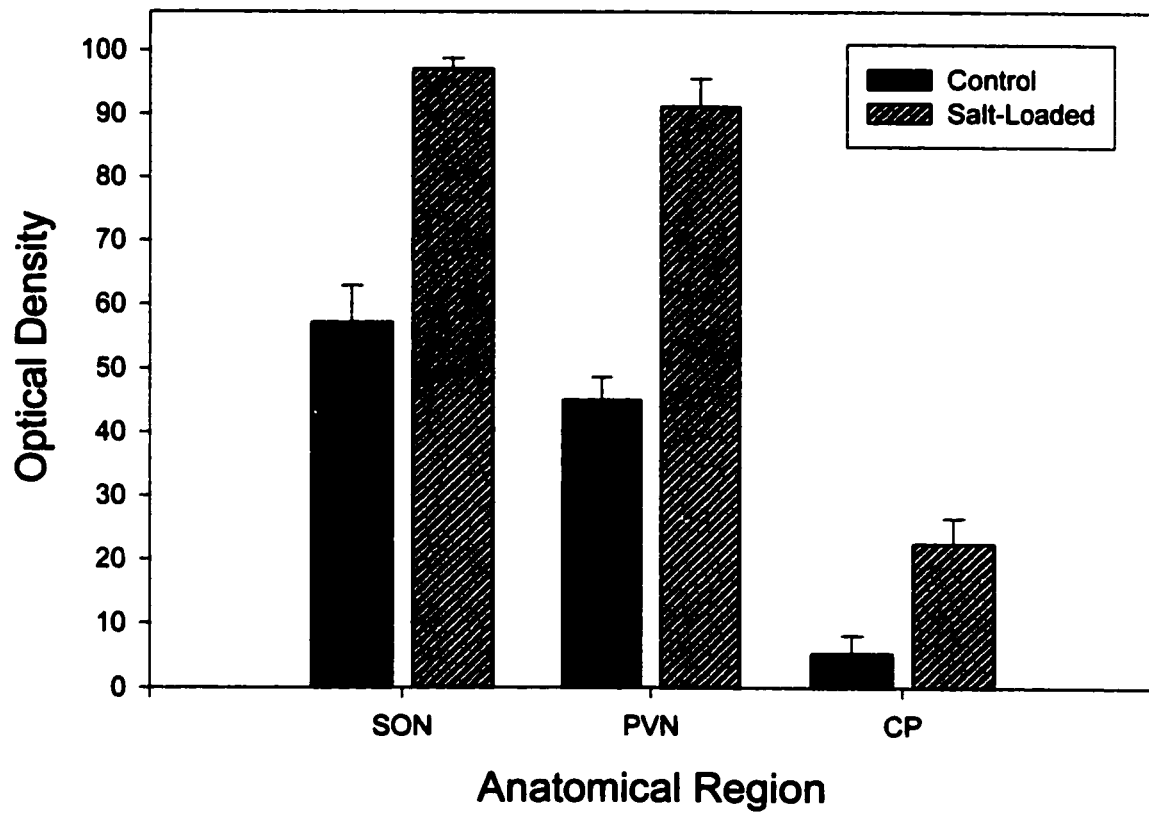


Fig. 2. Regulation of vasopressin mRNA in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus, and in the choroid plexus (CP) due to salt-loading for seven days. Significant increases ($p<0.05$) were seen in all salt-loaded tissues ($n=6$) when compared to the control group ($n=6$). Vasopressin mRNA levels, measured as optical density, increased 170% and 203% in the SON and PVN of salt-loaded animals, respectively. In the CP, vasopressin mRNA levels increased 423% in the salt-loaded group.



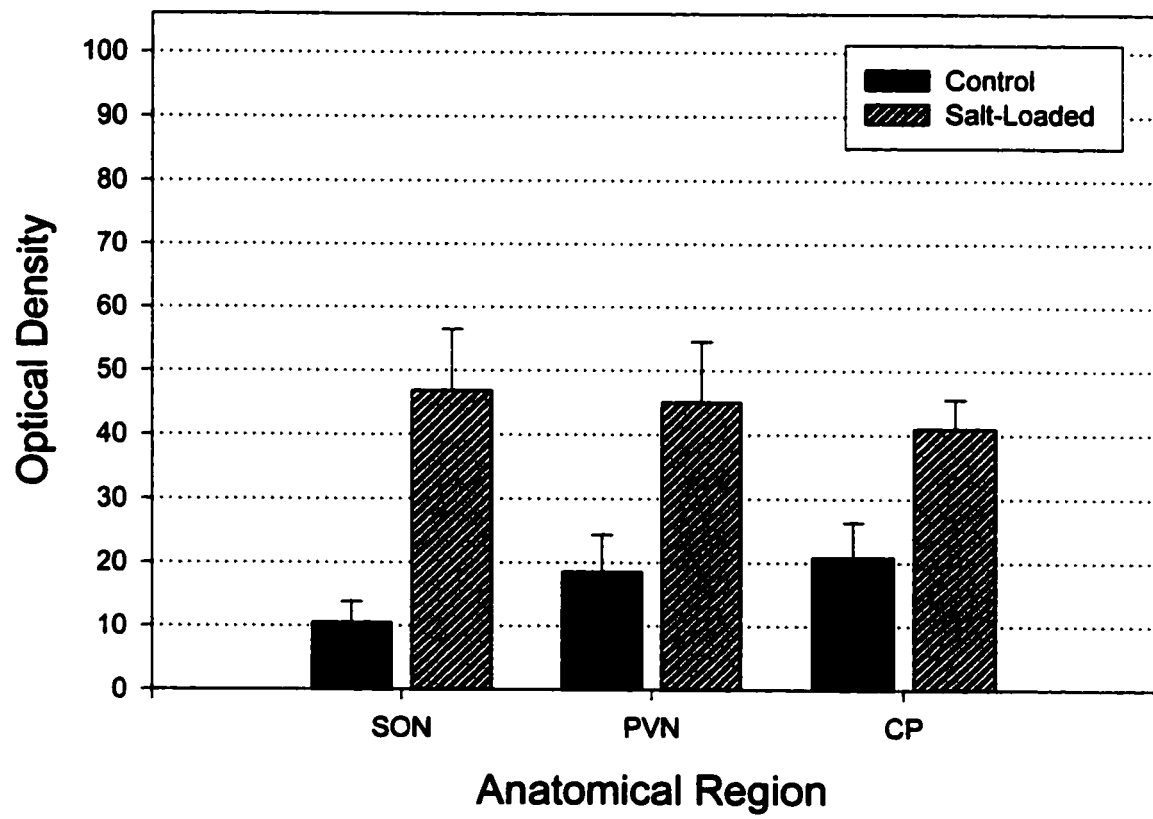
cells labeled with AVP mRNA in the choroid plexus (Figure 1B, compared to control, Figure 1A).

Vasopressin 1b receptor ($V_{1b}R$) mRNA levels in the SON, PVN, and the choroid plexus was increased by salt-loading animals (Figures 1 & 3). Compared to the control group, salt-loaded rats had significant ($p < 0.05$) increases in $V_{1b}R$ mRNA of 446% and 243% in the SON and PVN, respectively. Almost all magnocellular neurons of the SON and PVN from salt-loaded animals exhibited an increase in the number of $V_{1b}R$ transcripts (Figure 1D, F) compared to control animals, where although most the neurons were labeled, it was to a lesser extent (Figure 1C, E). Therefore, $V_{1b}R$ mRNA can be detected in both vasopressinergic and oxytocinergic neurons of control and salt-loaded animals. Few epithelial cells of the choroid plexus from control animals were labeled with $V_{1b}R$ mRNA (Figure 1G). In contrast, salt-loading caused an increase in the number of labeled choroid plexus epithelial cells and a 197% increase in the amount of $V_{1b}R$ mRNA (Figure 1H & 3).

Discussion

Brain water and electrolyte homeostasis is vital to maintenance of normal CNS function. Several processes are known to maintain electrolyte and water content within limits that permit continued normal brain function during plasma hypernatremia. In addition to transport of electrolytes and fluid by capillary passage (blood-brain barrier), important fluid and solute exchanges are mediated by the blood-CSF barrier of the choroid plexus (for review see Go, 1997). The choroid plexus actively takes up Na^+ (Davson & Segal, 1970) and Cl^- (Johanson et al., 1999) from plasma and transports them into the

Fig. 3. Regulation of vasopressin 1b receptor mRNA in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus, and in the choroid plexus (CP) due to salt-loading for seven days. Significant increases in the mean optical density ($p < 0.05$) were seen in all salt-loaded tissues ($n=7$) when compared to the control group ($n=8$). In the SON and PVN of salt-loaded animals optical density levels, as a measure of mRNA levels, increased 446% and 243%, respectively. Salt-loading increased the levels of vasopressin 1b receptor mRNA 197%.



CSF, resulting in the passive diffusion of water into the CSF (Cutler & Spertell, 1982; Go, 1997). During acute exposure to hyperosmotic conditions, the central nervous system exhibits a net loss of water content, and dramatically increases its uptake of Na^+ , Cl^- , and K^+ to counterbalance water loss from the salt load (Cserr et al., 1987). In spite of a dramatic increase in plasma osmolality (~ 263 to ~ 292 mOsmol/kg in the present study) after 7 days of salt loading, brain water content exhibited a minimal, albeit significant, level of reduction (from $\sim 79\%$ to $\sim 78\%$). Several other reports found nonsignificant decreases in brain water content (Holliday et al., 1968; Lien et al., 1990; Arief et al., 1997).

Increases in AVP and V_{1b} receptor mRNA levels in the choroid plexus after a reduction of brain water content from salt loading suggests vasopressinergic signaling is a component of a neuroendocrine feedback loop for maintenance of normal CNS fluid balance (Nilsson et al., 1992). Vasopressin has previously been suggested to play a role in brain water balance. AVP, for example, causes morphological changes in the choroid plexus epithelium that are similar to changes seen in other epithelial tissues involved in a water transport (Schultz et al., 1977; Liszczak et al., 1986; Nilsson et al., 1992), and AVP decreases the overall formation rate of CSF (Faraci et al., 1988; Szmydynger-Chodobska et al., 1990; Chodobski et al., 1998a). Peripheral hypernatremia, however, does not significantly alter CSF levels of AVP (Mens et al., 1980; Wang et al., 1982; Coleman & Reppert, 1985; Sorensen & Hammer, 1985; Chodobski et al., 1998b). These results suggest local synthesis of AVP by the choroid plexus may have a local effect, perhaps upon the very cells that synthesize the hormone or on adjacent choroid cells.

We presently have no direct information concerning the molecular processes

mediating AVP's effects in the choroid plexus. Work in other tissues indicates V_{1b} is a G-coupled ($G_{q/11}$ class) receptor stimulating phosphoinositol and intracellular Ca^{2+} pathways (Lolait et al., 1995; Rabadan-Diehl & Aguilera, 1998; Schoneberg et al., 1998; Chen et al., 2000). Investigations of several cell lines suggests AVP activation of the phosphoinositide pathway (Eveloff & Warnock, 1987; Harris et al., 1996) and Ca^{2+} (Lynch et al., 1986; Anwer & Atkinson, 1992; Casavola et al., 1992; Ishikawa et al., 1992) alters cellular Na^+ concentration or activates Na^+ transporters.

Vasopressin secreted by parvocellular neurons of the PVN maintains pituitary corticotroph responsiveness thereby insuring adrenocorticotrophic hormone (ACTH) release during periods of chronic stress (Aguilera, 1994). Pituitary corticotroph levels of the V_{1b} receptor are upregulated or downregulated in response to acute or chronic osmotic stress, respectively (Aguilera et al., 1994; Aguilera & Rabadan-Diehl, 2000). In contrast, there was no change in V_{1b} receptor mRNA levels during salt-loading (Rabadan-Diehl et al., 1995). Here we report upregulation of V_{1b} receptor message in the choroid plexus and magnocellular neurons of the SON and PVN after salt-loading.

The recent localization of AVP mRNA and immunoreactive AVP in the choroid plexus is suggestive of local autocrine or paracrine functions. The parallel changes in AVP and V_{1b} receptor mRNA levels we observed in the SON suggests similar ultrashort-loop feedback mechanisms are coordinated in neurohypophyseal neurons (Ludwig, 1998). Intranuclear release of AVP from application of hypertonic saline by microdialysis is blocked by a V_1/V_2 receptor antagonist (Wotjak et al., 1994). Further studies are needed to determine if V_{1b} receptor activation on SON neurons, coupled with the activation of

second messenger systems, is involved in transcriptional regulation of the AVP gene and/or is involved in neuronal firing patterns.

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Chapter Four

Discussion

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Vasopressin's Evolutionary Role in Osmoregulation

The ability of multicellular organisms to control their internal environment is dependent upon many complex processes. Paramount among these demands are requirements related to water and electrolyte balance, where interstitial and cellular water, ion, and other chemical constituents must be maintained within narrow ranges. For multicellular organisms, this process is indeed complex, since osmotic homeostasis, perhaps the oldest evolutionary precondition for life (Strange, 1994), must first maintain *cellular* integrity as a requisite to *multicellular* homeostasis. For multicellular organisms the "osmotic challenge" takes on wholly new levels of organization, calling upon processes coordinated by the endocrine and central nervous systems, gastrointestinal tract, and kidneys (Hill, 1990). The plethora of creatures of our world, in so many diverse and extreme habitats, is testimony to the success of osmoregulation.

Evolution of the vasopressin (and oxytocin) superfamily of genes has played an integral role in sea and land adaptation. Invertebrate organisms as ancient and varied as coelenterates [hydra: (Grimmelikhuijzen et al., 1982)], gastropods [the land-living mollusks: (Cruz et al., 1987)], beetles (Veenstra et al., 1984), and segmented worms (Satake et al., 1999) utilize vasopressin-like peptides. The presence of a single hormone peptide, vasotocin, that gave rise to two hormones (vasopressin and oxytocin in mammals) is believed to have arisen with the appearance of the world's first vertebrates, *Cyclostomata* [jawless fishes: (Acher et al., 1995)]. Oxytocin-like peptides originally mediated urea-based osmoregulation in cartilaginous fishes, but were relieved of this

function for its present role in mammals (Acher et al., 1995). Vasopressin, however, retained its most ancient function; control of multicellular osmoregulation. Its synthesis and release from neurons of the supraoptic and paraventricular nuclei of the hypothalamus is responsive to many inputs, but most importantly osmotic regulation of antidiuretic functions by the kidney.

Although classically considered in terms of its antidiuretic role, investigations of the past decade have called attention to vasopressin's many central nervous system functions. Vasopressin plays a crucial role in circadian rhythms mediated by the suprachiasmatic nucleus (Jin et al., 1999), stress-related endocrine functions of the adenohypophysis (Harbuz & Lightman, 1992), and autonomic functions mediated by vasopressinergic neurons in the amygdala (Hallbeck et al., 1999) and parvocellular subregion of the paraventricular nucleus (Sawchenko & Swanson, 1982). The latter system sends projections to brainstem autonomic centers (parabrachial region, nucleus of the solitary tract) and intermediolateral neurons of the spinal cord.

The cloning and *in situ* localization of vasopressin, and as many as five vasopressin receptors in widespread regions of the central nervous system, further expanded our knowledge of this fascinating hormone. Behavioral neurobiologists are investigating the role of vasopressin (and oxytocin) in diverse species-specific behaviors including memory, stress, fever, parental behavior and intraspecies affiliation (Bock et al., 1994; Ma et al., 1997a, b; Born et al., 1998; Nelson & Panksepp, 1998; Young et al., 1999). The localization of vasopressin receptors on vasopressinergic neurons of the supraoptic and paraventricular nuclei has stimulated the interest of neurophysiologists to

understand the mechanisms of autoregulation (Kombian et al., 1997; Ludwig, 1998; Dayanithi et al., 2000). Localization of vasopressin activity and vasopressin receptors on cells of the choroid plexus has further stimulated great interest in this peptide as an autoregulatory signal for water balance within the central nervous system, perhaps recapitulating its renal diuretic functions.

These latest discoveries provoked my keen interest in the potential unitary role of this hormone, which again emerges as a key player in homeostasis. I have explored two aspects of osmotic regulation of the vasopressinergic system: vasopressin transcription after acute, single and repeated stimulation of the supraoptic nucleus by hypertonic saline, and regulation of vasopressin and vasopressin receptor type 1b gene expression in the supraoptic and paraventricular nuclei and the choroid plexus after long term osmotic stimulation.

Stimulus-Transcription Regulation of the Vasopressin Gene

When vasopressin is released by magnocellular neurons, the production of more hormone by the neuron, through increases in vasopressin mRNA (increased transcription) and eventual translation of the mRNA into polypeptide, is necessary to replace stores in the nerve terminal. Research has clearly shown that acute increases in plasma osmolality result in not only the release of vasopressin from the nerve terminal, but also in transcription of the vasopressin gene (Herman et al., 1991; Shoji et al., 1994; Arima et al., 1999; Ma & Aguilera, 1999). Recently, it was shown that very small acute increases in plasma osmolality cause an increase in vasopressin hnRNA as quickly as five minutes after the stimulus (Arima et al., 1999). I found vasopressin hnRNA levels begin to increase

fifteen minutes after an injection of hypertonic saline, with hnRNA levels reaching their highest after thirty minutes (Figure 1, page 38). These results are in accordance with previously published results and further strengthen the temporal relationship between the sudden inception of plasma hyperosmolality and an increase in vasopressin gene transcription. The molecular processes causing the reduction of vasopressin hnRNA levels after a single acute injection of hypertonic saline has not been studied extensively. However, I found that vasopressin hnRNA levels return to normal levels 180 minutes after injection (Figure 1, page 38). Other researchers reported a similar time course (Herman et al., 1991; Shoji et al., 1994; Arima et al., 1999; Ma & Aguilera, 1999).

We define our double-injection paradigm as acute since the second injection was administered within 3 hours of the initial treatment. Although the present work did not include electrophysiological recording to verify that a second administration of hypertonic saline elicited a vigorous increase in magnocellular neuron activation, previous studies by Leng (1979) and Bourque (1989) demonstrated repeated exposure to hypertonic saline produces immediate, reversible, and dose-dependent changes in neuron activity. There has been just one previous report where vasopressin hnRNA levels were determined after repeated hypertonic saline treatment. Ma and Aguilera gave rats a daily ip injection of hypertonic saline for fourteen days, and on the fifteenth day the animals were sacrificed 15, 30, or 240 minutes after another injection of hypertonic saline (Ma & Aguilera, 1999). In that chronic paradigm, rats had a significant increase in AVP hnRNA in the SON after 15 minutes, but in contrast to other acute injection studies (Herman et al., 1991; Shoji et al., 1994), the rats had decreased AVP hnRNA levels after 30 minutes (Ma & Aguilera,

1999).

In my study a second acute increase in plasma osmolality 180 minutes after an initial increase in plasma osmolality resulted in increased levels of vasopressin hnRNA, but the increase was not significantly more than levels detected after a single injection of hypertonic saline. Based upon the findings of Brimble and Dyball (Brimble & Dyball, 1977), I predicted the second injection of hypertonic saline would have elevated vasopressin hnRNA levels (Figure 1, page 38) to values that were significantly greater than after a first injection. However, the lack of a greater increase in vasopressin hnRNA levels after a second injection may be because the second stimulus was presented when the neurons had already completely returned to baseline levels of electrical activity. Brimble and Dyball presented their second stimulus 60 minutes after the initial hypertonic infusion, and at this time plasma $[Na^+]$ would not have returned to baseline levels (Wang, et al., 1997). In the case of Brimble and Dyball, then, the response pattern they observed may be the result of the additive, almost dose-dependent, effects of the two hypertonic saline treatments. A similar "additive" effect is evident in the work of Leng (1979). Two boluses of 4M NaCl were infused (~90 seconds each) in the region of the supraoptic nucleus to anesthetized rats. The infusions were separated by ~120 seconds. Unit activity after the first and second infusions appears to have elicited the same vigorous level of activity, and there was a partial cessation of high neuronal activity between infusions. The duration of the high activity after the second infusion, however, was sustained and continued beyond the infusion period. The time courses used to measure vasopressin hnRNA after single and repeated intraperitoneal injections of hypertonic saline are in line

with findings that this stimulus elicits a rapid electrophysiological response and a robust alteration in vasopressin hnRNA levels. (Changes in the levels of hnRNA, however, probably lag behind initial neuronal activation on the scale of some minutes.) The present work is not inconsistent, then, with the working model that a relationship exists between neuronal activity and transcription regulation. More study is needed, perhaps using *in vitro* slice preparations where one can closely control osmotic events, to more fully appraise this hypothesis.

Vasopressin-Mediated Adaptation to Long-Term Hypernatremia

Chronic osmotic stimulation of vasopressin gene transcription results in increased vasopressin mRNA levels. Some researchers have reported substantial increases in vasopressin mRNA levels due to salt-loading. Sherman and colleagues reported that salt-loading for six days resulted in a 7.3-fold increase in vasopressin mRNA in the SON, and a 5.1-fold increase in the PVN (Sherman et al., 1986). My results are in line with many other researchers who looked at levels using *in situ* hybridization. Salt-loading for six to ten days results in a two-fold to three-fold increase in AVP mRNA in the SON (Zingg et al., 1986; McCabe et al., 1990; Suemaru et al., 1990). Salt-loading for 14 days results in similar increases in AVP mRNA in the SON and PVN (Burbach et al., 1984; Van Tol et al., 1987). Here I report a 170% and 203% increase in vasopressin mRNA in the SON and PVN, respectively, due to seven days of salt-loading (Figure 2, page 60).

Regulation of the vasopressin gene transcription in response to chronic stimulation may be partially dependent upon autoregulatory mechanisms.

Vasopressinergic neurons alter their firing patterns in response to chronic stimulation by

either acquiring or reinforcing a phasic pattern (Brimble et al., 1978). However, vasopressinergic neurons do not display increases in the number and extent of direct neuronal appositions or in the number of presynaptic terminals contacting two neurosecretory cells simultaneously ("double synapses"), like oxytocinergic neurons do, in response to chronic osmotic stimulation (Chapman et al., 1986). The control of vasopressinergic neuronal firing patterns has been shown to be influenced by vasopressin released by the same neurons. Investigators have suggested local release arising from depolarization acts as an autoregulatory feedback mechanism (Ludwig, 1998). Local, *dendritic* release of vasopressin is different from *axonal* release. Dendritic release occurs in response to many different stimuli but exhibits some spatial and temporal differences, and is primarily involved in controlling the electrical activity of magnocellular neurons. Autoregulation of neuronal activity has been demonstrated in other neuronal populations and therefore appears to be a general phenomenon (Ludwig, 1998).

I predicted that salt-loading would increase vasopressin receptor type 1b mRNA levels in the SON. Salt-loading for seven days resulted in a 446% and 243% increase in vasopressin receptor 1b mRNA in the SON and PVN, respectively (Figure 3, page 62). The greater increase of vasopressin receptor 1b mRNA in the SON could demonstrate that the SON has a more prominent role in the control of osmoregulation, but receptor autoradiography or other quantitative studies need to be performed to verify that role. The increase seen in both nuclei supports the views of Kombian and Dayanithi, that vasopressinergic neurons might be autoregulated through the local release of vasopressin and its binding to vasopressin receptor type 1b (Kombian et al., 1997; Dayanithi et al.,

2000). At present, the precise role of vasopressin receptor type 1b in the SON is not known. Other vasopressin receptor types may have a role in controlling vasopressinergic firing patterns during chronic osmotic stimulation, so the increase in vasopressin receptor type 1b due to salt-loading reported here might only be a small piece of a much larger puzzle. At the present time, these results show that the vasopressin receptor type 1b gene is upregulated during chronic osmotic stimulation and is probably involved in modulating vasopressinergic firing patterns. Further studies need to be performed to determine the effect of salt-loading on the other vasopressin receptor types, and to further determine the role of vasopressin receptor type 1b in magnocellular autoregulation.

Vasopressin and Vasopressin 1b Receptor Gene Expression in the Choroid Plexus

Vasopressin's osmoregulatory function throughout the animal kingdom substantiates the idea that it plays a role in CNS osmoregulation. The choroid plexus is responsible for the majority of CSF production, and although it is actually a secretory epithelium, it has been called a "kidney" for the brain (Spector & Johanson, 1989). This analogy is derived from the role the choroid plexus plays in CNS osmoregulation through the production of CSF. The transport of sodium across the choroid plexus epithelial cells drives the production of CSF (Johanson et al., 1990), and the main source of sodium entry into the brain during experimental hypernatremia is through the choroid plexus (Pullen et al., 1987). In the present study, a dramatic increase in plasma osmolality (~263 to ~292 mOsmol/kg) after 7 days of salt loading caused a minimal, albeit significant, reduction in brain water content (from ~79% to ~78%). This finding is in contrast to several other reports that found nonsignificant decreases in brain water content due to hypernatremia

(Holliday et al., 1968; Lien et al., 1990; Arieff et al., 1997). Reasons for this discrepancy could be species-related toleration of higher CNS osmolality or regulation of CSF production.

Vasopressin's presence in the central nervous system has previously been suggested to play a role in brain water balance. Morphological changes in the choroid plexus epithelium similar to changes seen in other epithelial tissues involved in a water transport are caused by AVP (Schultz et al., 1977; Liszczak et al., 1986; Nilsson et al., 1992). Central AVP also decreases the overall formation rate of CSF (Faraci et al., 1988; Szmydynger-Chodobska et al., 1990; Chodobski et al., 1998a). Peripheral hypernatremia, however, does not significantly alter CSF levels of AVP (Mens et al., 1980; Wang, B. C. et al., 1982; Coleman & Reppert, 1985; Sorensen & Hammer, 1985; Chodobski et al., 1998b). These results suggest local synthesis of AVP by the choroid plexus may have a local effect, perhaps upon the very cells that synthesize the hormone or on adjacent choroid cells. The 423% increase in AVP and 197% increase in V_{1b} receptor mRNA levels in the choroid plexus (Figure 2, page 60; Figure 3, page 62) after a reduction of brain water content from salt loading suggests vasopressinergic signaling is a component of a neuroendocrine feedback loop for maintenance of normal CNS fluid balance (Nilsson et al., 1992).

The study of how sodium transport is controlled by the choroid plexus is key to the development of new therapeutic approaches to treat diseases whose hallmark is the overproduction (edema) or underproduction (myelinolysis) of CSF. Increased hypernatremia has been linked to brain myelinolysis (Soupart et al., 1996). Faraci and

colleagues (Faraci et al., 1990) have shown that intracerebroventricular injection of AVP reduces choroid plexus CSF production by 35%. AVP may also play a role in cerebral edema (Faraci et al., 1990, 1994). Brain injury-induced edema is substantially reduced by the administration of V_1 receptor antagonists (Rosenberg et al., 1990; Nagao et al., 1994; Ikeda et al., 1997; László et al., 1999). Therefore, therapeutic alteration of CSF production through the use of vasopressin and/or vasopressin receptor analogs could improve the outcome of certain diseases.

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